

**THE ISOLATION, CULTURE, AND USE OF MARINE COPEPODS IN
AQUACULTURE**

5 This application claims priority to provisional application serial no. 60/416,535 filed
on October 8, 2002, entitled 'THE ISOLATION, CULTURE, AND USE OF
MARINE COPEPODS IN AQUACULTURE,' the entire contents of which is
incorporated herein by reference.

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NA16FY2812 awarded by the U.S. Department of Commerce, National Oceanic and
10 Atmospheric Administration, National Marine Fisheries Service. The government has
certain rights in this invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention relates to marine larviculture.

15 BACKGROUND INFORMATION

The use of cultured calanoid copepods has enabled recent breakthroughs in the
larviculture of tropical lutjanid snappers and groupers (Ogle *et al.*, 2000, Toledo *et*
al., 1999, Schipp *et al.*, 2001) and the nutritional superiority of marine copepods
versus conventional live prey (rotifers, *Artemia*) has been confirmed (Shields *et al.*,
20 1999). *Acartia* sp. copepods have been found to be effective diets for larvae of
European turbot (Støttrup *et al.*, 1986, Urup 1994) and golden snapper (Schipp *et al.*,
2001) while extensively cultured copepods (mixed calanoid species) have been
successfully used to rear red snapper and red-spotted grouper (Ogle *et al.*, 2000 ,
Toledo, *et al.*, 1999 , Doi *et al.*, 1997).

Having identified the high dietary value of marine calanoid copepods, researchers and aquaculturists have been seeking to develop methods of mass culture. Until recently “extensive” rather than “intensive” copepod production techniques have predominated in this field (Liao *et al.*, 2001, Van der Meeren and Naas 1997). In the extensive or “mesocosm” technique, a mixed assemblage of wild plankton is enclosed in a large fertilized outdoor tank, pond or lagoon (Van der Meeren and Naas, 1997, Divanach and Kentouri 2000, Benetti 2001). Fish larvae are then added to the mesocosm and allowed to graze on the endogenous plankton population. Alternatively, appropriate-sized zooplankton may be harvested from the mesocosm and supplied to larvae in separate hatchery tanks (Van der Meeren and Naas, 1997, Shields 2001). “Conventional” prey (rotifers, *Artemia*) may also be added during the rearing process as the endogenous plankton population becomes exhausted.

The extensive culture approach has the advantage of requiring only simple rearing facilities and of offering species and size diversity to meet the fish larvae’s changing developmental needs. It is therefore especially useful in locations lacking technical resources or for new fish species for which exact dietary requirements are unknown (Dhert *et al.*, 1997). However, productivity using this method tends to be highly variable due to lack of control over plankton abundance/species composition or physical environmental conditions. The open nature of the rearing system also offers a pathway for introduction of fish pathogens, for example, viral nervous necrosis (Liao, et al, 2001). Some operators have therefore adopted a more sophisticated extensive rearing approach in which the mesocosm is inoculated with microalgae and zooplankton from indoor stock cultures rather than from wild sources. This method has been successfully used for large-scale culture of *Acartia* spp, as feed for European turbot larvae and golden snapper (Urup, 1994 , Schipp *et al.*,2001).

Indoor intensive culture systems offer an alternative means of producing large quantities of marine calanoid copepods as larval feed. Intensive culture systems utilize less water volume than extensive mesocosms and can operate independently of outdoor environmental conditions, although they require close control over dietary inputs and water quality (Støttrup *et al* 2000, McKinnon *et al* 2003). Støttrup *et*

al, (1986) successfully reared *Acartia tonsa* for multiple generations in experimental rearing tanks (250-400L volume) using the microalga *Rhodomonas baltica*. However adult *A. tonsa* densities in this system were limited to 50-100/L. Based on these and similar findings, it was initially questioned whether calanoid copepod species can attain sufficient population densities to allow economically viable intensive culture. This assumption has since been revised following the discovery that much higher culture densities (up to 5,000/L) and nauplius yields can be obtained for *Acartia* sp, *Gladioferens imparipes* and *Oithona* sp (Schipp, *et al.*, 1999, Payne and Rippingale 2001, Lipman *et al.*, 2001), *Bestiolina similes*, *Parvocalanus crassirostris*, and *Acartia sinjiensis* (McKinnon *et al.*, 2003).

There still remains a significant need for improvements in isolation, culture and use of copepods in aquaculture.

RELEVANT LITERATURE

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SUMMARY OF THE INVENTION

Novel intensive controlled larviculture methods are employed using copepod live feed that provides for enhanced survival and growth of a variety of fish larvae. The larvae are reared on a controlled feed composition of *Parvocalanus sp.*, grown separately and then added to the larvae or *in situ* with microalgae in a controlled environment under conditions to provide rapid growth and improved survival during the early stages of development. The calanoid copepods for marine fish aquaculture are cultured in controlled quantities, under biosecure conditions, and to a defined nutrient composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of a 2-phase intensive culture cycle with marine copepod *Parvocalanus sp.*;

Figure 2 shows comparative survival and growth rates of red snapper (*Lutjanus campechanus*) larvae receiving microalgae with/without addition of cultured rotifers or copepods, *Parvocalanus sp.*;

Figure 3a shows a 28-day-old flame angelfish (*Centropyge loriculus*); Figure 3b shows a 25-day-old red snapper (*Lutjanus campechanus*); and Figure 3c shows a 28 day-old bluefin trevally (*Caranx melampygus*);

Figure 4 is a graph of temporal changes in the densities of *Parvocalanus* sp adults and copepodites under controlled growth conditions;

Figures 5A-C are graphs of the temporal profile of copepod density; 5A in a 30,000L tank, Trial #1; 5B harvested from a 30,000L tank, Trial #1; and 5C from a 30,000L tank, Trial #2;

Figures 6A and 6B are bar graphs of mean percentage survival of larvae grown under predetermined conditions to day 7ph;

Figures 7A-C are bar graphs of: 7A, mean % feeding incidence; 7B, myotome height; and 7C, survival rate, of flame angelfish offered *Parvocalanus* sp at different densities; and

Figures 8A and 8B are graphs of mean standard length of flame angelfish and bluefin trevally, respectively, using a combination feed.

DETAILED DESCRIPTION OF THE INVENTION

Improved methods and materials are provided for aquaculture of difficult-to-rear marine fish and other aquatic animals. Cultured copepods are employed as live feed grown concomitantly *in situ* on microalgae in the presence of the fish larvae or grown independently in separate tanks and fed to the fish larvae, both under selected controlled growth conditions. Particularly, *Parvocalanus* sp is employed as the live feed. The *Parvocalanus* sp can be grown on a variety of individual or mixtures of microalgae and fed to the fish larvae, where a broader spectrum of microalgae feed may be used when the copepods are grown *in situ*.

Parvocalanus is a large family having a large number of species. Of particular interest is a species that can be found in the coral reef areas of Kaneohe Bay, Oahu, USA. The subject *Parvocalanus* sp. is readily and reproducibly isolated from Kaneohe Bay and the same or equivalent species may be found in other oceanic sources. In referring to *Parvocalanus* sp. it is intended to include the species employed in the subject invention and other *Parvocalanus* sp. having the same

desirable characteristics. The subject, tropical copepods are characterized by higher culture production densities than other tropical or temperate copepod species, with the subject species reaching production levels of 3,750 nauplii/L/day in 400 – 800L batch culture systems harvested at 5 day intervals employing the conditions set forth in the Experimental section. In contrast, reported production rates for *Acartia* sp in 1,000L systems is 440 nauplii/L/day and for *Gladiferens imparipes* in 500L systems is 878 nauplii/L/day. Production of the subject species achieves 20eggs/adult/day, with production of over 40 offspring/female/day or higher. In contrast, the highest production achieved with *Parvocalanus crassirostris* was 31 offspring/female/day. Culture densities of the subject species reach an average 20 to 45 nauplii/ml or higher. Stage-specific differences in algae needs (*Chaetoceros* sp. and *Isochrysis* sp.) are evident for optimum growth, survival, and fecundity for the subject species. In contrast, no differences were observed in fecundity of *Parvocalanus crassirostris* fed different algae mixtures. Mean nauplis NI stage dimensions of the subject species is 75.6 μm in length x 41.3 μm in width compared to dimensions of 62.1 μm in length x 38.7 μm in width for *Parvocalanus crassirostris*. The subject species has proven effectiveness in improving growth and survival of small, tropical marine fish species including flame angelfish (*Centropyge loriculus*), bluefin trevally (*Caranx melampygus*), and red snapper (*Lutjanus campechanus*).

Under favorable conditions described herein, *Parvocalanus* sp. nauplii metamorphose to copepodites on day 4 post-stocking, with the first adult copepods observed 18-24 hours later. The adults *Parvocalanus* sp. then live for up to 9 days, representing a total life span of circa. 13-14 days in culture. They can be grown with a variety of microalgae, such as *Isochrysis* sp, *Chaetoceros* sp, *Tetraselmis* sp, and *Nannochloropsis* sp, individually or together, as well as other species depending upon the conditions under which they are employed, whether grown *in situ* with the fish larvae or grown independently in the absence of the fish larvae. Generally, the total cell density of the microalgae will be maintained at or about in the range of about 10^5 to 10^7 ml^{-1} , usually in the range of about 10^5 to 10^6 ml^{-1} . Usually, the fecundity of the

Parvocalanus sp should be at least about 15 eggs/adult/day, preferably at least about 20 eggs/adult/day.

Generally, the nauplii will be presented to fish larvae in tanks or ponds of at least 200-liter capacity, but usually between 1,500 and 100,000-liter capacity, at a density of at least about 0.5 ml⁻¹, more usually at least about 5ml⁻¹, and may be as high as 20ml⁻¹ or higher, usually not higher, and maintained at that level during the course of larval rearing. Target densities are reached and maintained either through daily, supplemental additions of nauplii, as required, through the 2-phase copepod production system described herein, or through natural recruitment when grown *in situ* with the fish larvae, or a combination of both. At target levels, larvae of fish species not only ingest the nauplii, but survive beyond yolk exhaustion on the diet and grow rapidly. The *Parvocalanus* sp nauplii are an efficient and effective diet, particularly during the early phase of larval rearing, as compared to other common diets such as rotifers (*Brachionus* sp). The early phase begins at day 1 of the larvae and continues to day 5, usually day 8, and more usually day 10. In the subsequent later phase, *Parvocalanus* sp feeding is desirable, but less important, and may replace use of *Artemia*.

The rearing method employs substantially sterile conditions, using sterilized seawater or synthetic water and the larvae are reared under conditions that at least substantially inhibit the introduction of infectious agents. All of the components used in the rearing are selected or treated to be substantially free of contaminants and infectious agents.

Different protocols may be used to raise marine fish larvae using the *Parvocalanus* sp. Larvae are usually stocked directly or as fertilized eggs into tanks or ponds resulting in densities of usually 1 to 30 one-day-old larvae/L. Additions of *Parvocalanus* sp. will usually be daily, but may be made more or less frequently. *Parvocalanus* sp. nauplii added to the fish larvae may be advantageously raised separately in a 2-phase culture system described herein. Nauplii are first stocked in at least 600-liter vessels, usually between 1,000 and 100,000-liter vessels, and fed

microalgae, individual species or combinations of species, exemplary are *Isochrysis*, sp. and *Chaetoceros* sp, and usually at at least about 150,000 cells/ml, more usually at 200,000 cells/ml or more, and may be at 10^7 , usually not more than about 10^6 , cells/ml of either or each. Other microalgae that find use include *Tetraselmis* sp, etc.

5 For the method employing separate growth of the *Parvocalanus* sp, namely a 2-phase system, when nauplii reach adult stage and begin to reproduce, resulting nauplii are harvested from the vessels and fed to larvae. Adults are reintroduced to the 2-phase copepod culture system to reproduce again until they reach the end of their life span.

10 Alternatively, the nauplii may be produced *in situ*, in effect 1-phase, by combining in the same environment, e.g. tank, the microalgae, the *Parvocalanus* sp and the fish larvae. *Parvocalanus* sp. grown *in situ* are first stocked as adults usually at least 1/ml, but no less than 0.05/ml in the presence of microalgae, desirably *Isochrysis* sp. or other microalgae, solely or in combination, which should be at a total concentration of between about 10^5 – 10^6 , more usually 100,000 - 500,000, cells/ml, particularly
15 200,000-400 cells/ml, one day prior to stocking fish larvae, and the level maintained in that range. Adults and copepodites are maintained at targeted levels usually 1-2/ml or higher of each through natural recruitment. Supplemental additions of nauplii may be provided daily, as required, to maintain targeted naupli densities, so that a separate 2-phase copepod culture system may be necessary.

20 In both approaches, the environmental conditions of the larval rearing tanks are substantially the same, and target densities of copepod nauplii are between 0.5 and 10/ml, but usually 1 to 5/ml. *Isochrysis* sp. levels in larval rearing tanks are maintained usually between 100,000 and 500,000 cells/ml, if necessary, through daily additions from stock cultures. Salinity of the larval rearing water is usually between
25 15 and 35 ppt, usually about 30 to 35 ppt, with the pH at least about 7.0, and usually between 7.5 and 8.5, but no greater than 9.0. Daily water replacement will usually not exceed 20-30% daily with copepod feeding. The temperature is maintained in the range of about 20-30°C, usually between about 23°C and 28°C, and dissolved oxygen levels usually are at least 5.0 ppm, but more usually between 5.0 and 6.5 ppm. The

water conditions may be tested twice daily, but will usually be tested at least once every two days. Generally, initially fish larvae will be stocked in an amount of at least about 10 fish larvae L⁻¹, usually at least about 20L⁻¹, more usually at least about 25L⁻¹, and not more than about 100L⁻¹, usually not more than about 75L⁻¹. Under these conditions fish larvae can be raised to at least 18 days of age, desirably 25 days of age, and can go to 40 or more days of age, in relation to the particular fish species. The fish and fish larvae are then harvested from the larval rearing tanks through a center drain and into a harvest cradle with usually between 500 and 1,000 micron mesh screen, more usually about a 700 micron mesh screen

Fish that may be grown according to the subject invention include fish for human consumption, e.g. fish in the taxonomic families Lutjanidae (snappers), Etilinidae (deep water snappers), Carangidae (jacks), Serranidae (groupers, sea basses, sea perches), Mullidae (goatfishes), Polynemidae (threadfins), Rachycentridae (cobias), Coryphaenidae (dolphin fishes), Sciaenidae (drums), Scrombridae (tunas) and ornamental fish, e.g. fish in the taxonomic families Pomacanthidae (angelfishes), Chaetodontidae (butterfly fishes and related families), Acanthuridae (surgeonfishes and tangs), Pseudochromidae (basslets), Zaclidae (Morrish idol), Labridae (wrasses), Pomacentridae (damsel fishes and anemonefishes), Gobioidae (gobies), Cirrhitidae (hawkfishes), Syngnathidae (pikefishes & seahorses), and families in the suborder Blennioidei (blennies).

A 2-phase intensive copepod mass culture system is shown in Figure 1. This system involves the daily harvest of *Parvocalanus* sp nauplii (as prey for fish larvae) and recruitment of new *Parvocalanus* sp adults in separate environment-controlled culture vessels 3, 5. Master cultures of adult *Parvocalanus* sp. are maintained separately, in usually 15-liter kreisels at usually between about 20 and 30°C, usually about 24°C and usually between 2 and 7 adults/ml, usually 5 adults/ml, that usually contain 10 – 12 nauplii/ml for inoculation and restart of the 2-phase system with resulting nauplii. Copepods in the master cultures are usually fed the same algae at the same concentrations and conditions as described in the 2-phase system below.

In the 2-phase system, the copepods are fed microalgae, one or more species, e.g. 2, generated in closed photobioreactors 7, 9. The preferred conditions for the photobioreactors are: pH between 7 and 8, but no greater than 9.0; dissolved oxygen no less than 5.0, generally between 5 and 6.5 ppm; temperature between 20 and 26°C, usually about 24°C, and salinity between 15 and 35 ppt, but usually about 32 ppt. Microalgae, usually *Chaetoceros* sp. and *Isochrysis* sp., and usually between 100,000 and 1,00,000 cells/ml, but at least 100,000 cells/ml, and usually between 100,000 and 300,000 cell/ml for each algae, are metered 13, 15, 17, 19 from the bioreactors into the nauplius production (NP) tank 3 and broodstock recruitment (BR) tank 5 at the appropriate concentration and species ratio for each copepod developmental stage, nauplius, copepodite, and adult. The concentrations of algae used for each developmental stage are usually the same. Concentrations of algae maintained in NP and BR tanks are between 200,000 and 400,000 cells/ml total, usually 300,000 cells/ml, and usually between 100,000 and 200,000 cells/ml of each algae, usually 150,000 cells/ml of each. The species of microalgae from bioreactor 7 or 9 used for copepod culture can be altered to match the nutritional requirements of different species of marine fish larvae.

The number of microalgae species used can also be modified with addition or deletion of other bioreactors. The amino acid, fatty acid, and proximate composition (content of lipid, protein, ash, and nitrogen-free extract) profiles of the algae used influences the profiles in copepods, their growth, survival, and fecundity. The resulting profiles in copepod nauplii, which are consumed by the fish larvae, in turn influence the profiles in fish larvae, their growth and survival. Algae are chosen with profiles that optimize copepod growth, survival, and fecundity and fish larvae growth and survival. A minimal quantity of culture water is withdrawn during nauplius harvest 21 to lessen microalgae usage. Nauplii are preferentially harvested with the use of special techniques, usually light traps that naturally separate adults and nauplii *in situ*. Nauplii and any non-separated copepodites and adults are then drain harvested into a 38-micron mesh sieve. This mix of copepods is then sieved through a 105-micron mesh sieve to retain adults and large copepodites, and harvest nauplii to feed

fish larvae. Nauplii are usually harvested when nauplii densities reach at least 5/ml, more usually between 10 and 20/ml, or even higher. Nauplii from the production tank 3 are repeatedly harvested 21 to feed 20 fish larvae. Nauplii from the production tank 3 are initially inoculated 23 from master cultures into the BR tank 5 at densities
 5 usually between 2 and 10 nauplii/ml, more usually about 5 nauplii/ml. Once the broodstock in tank 5 matures, the remaining contents of production tank 3 are discarded 24. The mature nauplii in tank 5 are transferred 25 to production tank 3.

Preferably, the production tank 3 and the BR tank 5 are interchangeable, inoculation line 25 is reversible and the production tank 5 has a harvest line 21 and discard line
 10 24 similar to tank 3. Instead of transferring mature nauplii from tank to tank, the functions of the tanks are interchanged. Tank 5 becomes the production tank and mature breeder nauplii are inoculated into tank 3, which becomes the BR tank.

When nauplii in tank 5 are mature, tank 3 is discharged 24. Tank 3 is filled and nauplii from tank 5 are inoculated into tank 3. Nauplii are repeatedly harvested from
 15 tank 5 and the harvest from tank 5 is transferred to feed 20 fish larvae through an outlet in tank 5 that mirrors outlet 21. When the nauplii in tank 3 are mature, the functions of the tanks are again reversed back to that shown in Figure 1. This modular copepod production system can be scaled up in a controlled fashion by adding further NP 3 and BR 5 tanks. The 2-phase system operates in biosecure mode to avoid
 20 contamination, utilizing sterilized sea-water (15 – 35 ppt), usually by autoclave or ultra-violet light, or artificial sea-water made in the lab, usually in closed-loop or in a biosecure, indoor culture room, usually with positive air pressure, and with controlled personnel access, usually with disinfection prior to entry and exit.

The subject methods permit the use of electronic database control and monitoring.
 25 Samples may be automatically taken from the various tanks, analyzed as to the population and the components of the water, and the information fed into the database. By employing a computer, the data can be analyzed and the conditions associated with each tank modified as appropriate. An algorithm is employed that

uses the information obtained from the samples to adjust conditions to provide for optimum copepod production and fish maturation.

The following examples are intended to illustrate but not limit the invention.

EXPERIMENTAL

5 Zooplankton were collected from shallow coral reef areas in Kaneohe Bay, Oahu, HI using an 50 cm diameter plankton tow net of 120_μm mesh aperture, towed down-current at low speed. The contents of each 5-10min tow were concentrated into separate sealed 15L plastic buckets and transported to an Oceanic Institute laboratory. The bucket contents were then size-fractionated using a 100_μm aperture nylon screen
10 in order to separate out adult copepods. Those organisms retained on the 100_μm screen were examined using an Olympus SZX12 stereomicroscope to identify copepod species, and adult stages for culture. Twenty adult individuals of 5 different copepod species (*Undinula vulgaris*, *Labidocera madurae*, *Acartia* sp, *Parvocalanus* sp and an unidentified cyclopoid species were then transferred by glass Pasteur
15 pipette into separate 250ml Erlenmeyer flasks containing filtered, UV-sterilized seawater. Four such flasks were stocked for each copepod species and each copepod species was offered the following microalgae: *Chaceteros* sp only, at a cell density of $1.5 \times 10^6 \text{ ml}^{-1}$; *Tetraselmis* sp, at a cell density of $1 \times 10^5 \text{ ml}^{-1}$; *Nannochloropsis* sp only, at a cell density of $6 \times 10^6 \text{ ml}^{-1}$; and the three together at cell densities of $5 \times 10^5 \text{ ml}^{-1}$,
20 $3 \times 10^4 \text{ ml}^{-1}$, and $2 \times 10^6 \text{ ml}^{-1}$, respectively.

The flasks were maintained under static, continuously lit (with fluorescent lamps), conditions at 25°C for 7 days, after which time copepod numbers, developmental stages and size distribution were quantified for each species. *Parvocalanus* sp exhibited the highest survival and fecundity rates of the isolated species and produced
25 nauplii with appropriate dimensions (mean nauplius length at hatch = 77.8 microns) for small marine fish larvae.

A series of experiments was carried out to compare different physical environments, microalgae concentrations and species ratios, copepod stocking densities and harvest

techniques for *Parvocalanus* sp. Stage-dependent differences in algae requirements were found. The *Parvocalanus* sp fecundity equaled the best-published figures for other cultured calanoid copepod species at more than 20 eggs/adult/day. High productivity was maintained on scale-up in 15L cultures with *Parvocalanus* sp nauplii densities of up to 45/ml being achieved. Copepods from these cultures were used to stock larger 600L rearing tanks. Reliable copepod population growth was achieved. Continuous production of microalgae in closed photobioreactors was achieved for reliable high algae quantities for the system scale-up.

1L glass beakers were used as rearing vessels in separate experiments to quantify feed uptake, survival and growth rates of flame angelfish (*C. loriculus*) and red snapper (*L. campechanus*) larvae receiving different concentrations of *Parvocalanus* sp nauplii. The fish larvae were stocked at a density of 50L⁻¹ into beakers containing *Isochrysis* sp microalgae (cell density 10⁵ml⁻¹). *Parvocalanus* sp nauplii were then offered to the larvae at a density of 1, 5, or 10ml⁻¹. Larvae of both fish species ingested the nauplii and survived beyond yolk exhaustion, as well as grew rapidly.

The advantage of *Parvocalanus* sp nauplii over other cultured live prey was shown by a comparison with rotifers (*Brachionus* sp) as prey for red snapper larvae. Red snapper larvae were stocked into 1L glass beakers containing *Isochrysis* sp microalgae (cell density 10⁴ml⁻¹) at a stocking density of 50 larvae L⁻¹. Both rotifers and *Parvocalanus* sp nauplii were offered at a prey density of 5 organisms ml⁻¹ and the larvae's survival and growth rates and feed incidence monitored. The superior survival and growth characteristics of *Parvocalanus* sp-fed red snapper larvae were clear. illustrated in Figures 2a and 2b. As shown in Figure 2a, *Parvocalanus* sp-fed larvae 31 exhibited a mean survival rate of 31.5% to day 7 post-hatch versus just 1.5% for rotifer-fed larvae 33. Also, the *Parvocalanus* sp-fed larvae 35 were more than 80% larger on average than rotifer-fed snapper larvae 37 by day 7 as shown in Figure 2b. The results of these laboratory feeding experiments were verified in large-scale fish rearing trials conducted with *Parvocalanus* sp mass culture.

As shown in Figures 3a-3c, larvae of flame angelfish 41, red snapper 43 and bluefin trevally 45 were all reared to metamorphosis using intensively cultured *Parvocalanus* sp. Larvae of each species were stocked into 1,500L or 4,000L rearing tanks containing *Isochrysis* sp microalgae at densities ranging from 1 to 5 larvae L⁻¹. The fish larvae were fed either by controlled daily additions of the appropriate *Parvocalanus* sp size class or by introducing adult *Parvocalanus* sp, which then produced nauplii in the rearing tank.

1.1 Scale-up of *Parvocalanus* sp cultures

Work was initiated to develop intensive mass copepod culture techniques in large volume containers. This work was carried out using square polyethylene tanks 100 x 100 x 60 cm in dimension. The culture tanks were continuously lit, gently aerated and maintained at a temperature of 27-28°C.

Initial trials were carried out at 100L or 200L working volume, using copepods supplied from 25L polycarbonate tanks. Culture volume was doubled each time adult copepod density reached 1ml⁻¹, up to a maximum volume of 400L, at which point a new culture tank was inoculated. A mixture of *Chaetoceros* sp and *Isochrysis* sp microalgae was supplied when available, although due to *Isochrysis* sp shortage, the cultures were sometimes fed only with *Chaetoceros* (cell density 300,000-500,000 ml⁻¹). Microalgae was added once per day to the desired cell density. Each culture tank was completely harvested at 3-day intervals. The harvested copepods were concentrated in a 38µm nitex mesh bag, rinsed and re-stocked into a clean tank.

During these initial trials, *Parvocalanus* sp nauplius densities of up to 30 ml⁻¹ were obtained although the cultures tended to be unstable, possibly due to inconsistency of microalgae supply. Routine harvesting of nauplii was not tested during this phase.

Microalgae production capacity was subsequently increased to enable routine feeding of the copepod cultures on a combination of *Chaetoceros* sp and *Isochrysis* sp. As overall copepod supply increased, the practice of splitting cultures once adult densities reached 1 ml⁻¹ was dropped without detriment to culture performance. Using

this approach, individual culture tanks could be maintained indefinitely without crashing. Figure 4 illustrates densities of *Parvocalanus* sp adults/copepodites and nauplii for one such tank, illustrating the characteristic cyclical fluctuations in copepod abundance.

5 1.2. Mass zooplankton culture using an extensive, mesocosm approach

Preliminary trials to mass culture copepods in large outdoor tanks were conducted. Four sequential zooplankton rearing trials were conducted in 20' diameter circular tanks with operating volume 30,000 L. The tanks were filled with seawater and inoculated with microalgae before stocking with zooplankton. Nutrients were added
10 prior to inoculation, at 10% of the concentration normally used for microalgae mass culture, i.e.: For 30,000L volume -

Ammonium sulphate, 300g
Monopotassium sulphate, 90g
Urea, 15g
15 Fe-EDTA, 30g
Trace metal mix, 3g

A water exchange rate of 10% day⁻¹ was typically used to avoid elevation of inorganic nitrogen levels. As a general guideline, further nutrients were added when
20 the concentration of total ammonia nitrogen fell below ~1mg L⁻¹.

Once a microalgae bloom had been established a mixed zooplankton population was stocked into each culture tank. The zooplankton was collected by plankton net from Kaneohe Bay and the 100-550µm size class introduced to the culture tank. The available types and quantities of zooplankton differed on each occasion. The two
25 predominant copepods in the 100-550µm size class were an unidentified cyclopoid species and *Parvocalanus* sp. Further microalgae was added to the culture tanks as required during copepod rearing.

Performance was highly variable among runs. Trial #1 yielded high quantities of copepods over a three-week period, sufficient to allow repeated harvesting of nauplii

as feed for fish larvae (Figs 5-1a&b). However, copepod productivity was much lower in trials 2-4 which were characterized by short-lived, low amplitude zooplankton blooms.

Reasons for the differences in productivity between trial #1 and trials 2-4 are unclear and are confounded by the sequential nature of the runs. It may be significant that the culture tank used for trial #1 was black in color, whereas those used for the remaining trials were white. Also, trials 2 and 4 experienced blooms of the diatom *Navicula* sp (unsuitable as a copepod diet) while copepod densities were still low. In contrast, the concentration of *Navicula* sp. in trial #1 only became elevated toward the end of the copepod production cycle.

In summary, trial #1 demonstrated the feasibility of mass culture of Hawaiian copepods using conventional extensive “mesocosm” procedures. The copepods generated in this trial were sufficient to conduct pilot scale rearing of flame angelfish and bluefin trevally larvae (see next section). While the high variability in culture performance is problematic and characteristic of such extensive zooplankton production systems, we conclude that this approach is worthy of application. In particular, the use of copepods from a defined source (i.e., indoor stock cultures), together with better control over water treatment and microalgae culture parameters, is indicative of a more reliable outdoor mass culture method.

2. Evaluation of cultured marine zooplankton as prey for small fish larvae

2.1. Small scale, “start-feeding” experiments

A series of replicated experiments was carried out to quantify the feeding incidence, survival and growth rates of fish larvae offered different types and concentrations of cultured zooplankton during the critical “first-feeding” phase. These small-scale experiments were carried out in 1L glass beakers and 25L polycarbonate tanks, maintained without water exchange.

The following standard experimental conditions were applied. The rearing containers were placed in a temperature-controlled water bath, adjusted to 26°C. Illumination

was provided by overhead fluorescent lamps, on a 12L:12D diurnal photoperiod. The 25L rearing containers were gently aerated using a single, central airstone, while the 1L containers did not receive any aeration. The rearing containers were filled with seawater from a header tank containing biofilter media. This water supply was continuously exchanged at a rate equivalent to 100% of total header tank volume per day. When required to fill the experimental rearing containers, seawater was drained from the base of the header tank via a UV-sterilizing unit.

A stocking density of 30-50 fish eggs L⁻¹ was used in all experiments. Numbers of hatched larvae and daily larvae survival rates were estimated in the 1L glass beakers by direct visual inspection, while 50ml water samples were taken from the 25L containers and the contents collected on a nitex screen for counting. Larvae for size measurement and gut contents analysis were pipetted from the rearing containers, anesthetized using MS222 and examined using an Olympus SZX12 stereomicroscope. Total numbers of surviving larvae were directly enumerated at the end of each experiment.

An initial experiment was carried out with flame angelfish larvae in 25L rearing containers, comparing the survival and growth rates of larvae receiving only *Tetraselmis* sp microalgae with those receiving *Tetraselmis* sp plus *Parvocalanus* sp at a density of 2 nauplii/ml (larvae experiment #3). Differences between the growth performance of the 2 diet groups were evident from an early developmental stage, such that *Parvocalanus* sp-fed angelfish larvae had a mean myotome height of 195.6 μm ($\pm 17.9 \mu\text{m}$) on day 5 post-hatch, compared to a myotome height of 138.7 μm ($\pm 11.8 \mu\text{m}$) for those receiving only *Tetraselmis* sp microalgae. Larvae survival rates were similar between the 2 diet groups to day ph, however those larvae offered only microalgae suffered rapid mortality during yolk exhaustion, with zero survivors remaining by day 7 ph. In contrast, *Parvocalanus* sp-fed flame angelfish larvae retained high rates of survival during the same critical period. Indeed, it was possible to continue rearing this group of angelfish larvae beyond the planned 7-day duration of the experiment, to 22 days ph.

Having obtained good preliminary confirmation of *Parvocalanus* sp's efficacy as a larval diet, further diet research focused on culturing and evaluating this copepod species. A subsequent diet experiment with red snapper larvae in 1L rearing containers (larvae experiment #4) compared rotifers versus *Parvocalanus* sp nauplii as prey. The superior survival and growth characteristics of *Parvocalanus* sp-fed snapper larvae are clearly illustrated in Figure 5B *Parvocalanus* sp-fed larvae exhibited a mean survival rate of 31.5% to day 7 ph, versus just 1.5% for rotifer-fed larvae (Figure 5A). Also, the *Parvocalanus* sp-fed larvae were more than 80% larger on average than rotifer-fed red snapper larvae by day 7 ph (Figure 6).

In an effort to optimize prey densities for fish larvae during first-feeding, a further small scale experiment (larvae experiment #5) was carried out comparing the survival and growth rates of flame angelfish larvae offered *Parvocalanus* sp nauplii at densities of 0, 1, 5, or 10 nauplii ml⁻¹.

Under the conditions tested, there was no discernible advantage of offering *Parvocalanus* sp nauplii to flame angelfish nauplii at densities greater than 1 ml⁻¹. Larvae receiving copepod nauplii at a density of 1 ml⁻¹ exhibited lower mean feeding incidence on day 5 ph, while all three copepod-fed groups displayed similar rates of feeding incidence by day 7 (Figure 7a). Significant differences in fish size (myotome height) were observed on day 5 ph, however these size differences were not proportional to the amount of food offered (Figure 7b). Larvae in the 5 ml⁻¹ diet group exhibited the lowest mean myotome height on day 5. Differences in mean size were not statistically significant on day 7 post-hatch. All diet groups experienced a sharp decrease in survival between days 4 and 5 ph (Figure 7c). No survivors were found in the microalgae-only group on day 7, while mean survival rates for the remaining copepod-fed groups ranged from 4.6% to 7.7% of hatched larvae and were not significantly different (Figure 7c).

2.2. Pilot scale larvae rearing trials

Having established that *Parvocalanus* sp is an appropriate first prey organism for small tropical marine fish larvae, it was next sought to test whether cultured calanoid

copepods can be used to rear larvae beyond first feeding to metamorphosis. This work was carried out in larger, 1,500L rearing tanks equipped with water exchange. Due to the limited quantities of *Parvocalanus* sp available from indoor monocultures at this stage of the project, the feed required for these larger rearing tanks was harvested from an outdoor “mesocosm” system. *Parvocalanus* sp was the dominant, but not exclusive copepod species harvested from this system.

Three larvae rearing trials were conducted using this approach, two with flame angelfish and one with bluefin trevally. In each case, the rearing tanks were inoculated with a combination of *Nannochloropsis* sp and *Tetraselmis* sp microalgae. Copepod nauplii were added at a density of 1.5-2.0 ml⁻¹ on day 2 ph. Residual copepod levels were then monitored daily and further nauplii added as required, to maintain a density of 2 ml⁻¹ or greater. Copepod nauplii were available daily from the mesocosm system until day 10 ph, and only intermittently thereafter. Rotifers were introduced from day 11 ph and rotifer densities of 3-5 ml⁻¹ subsequently maintained by daily addition. *Artemia* nauplii were first offered to larvae on day 19 ph and subsequently maintained at a density of 3-5 L⁻¹.

Using this approach, higher survival and growth rates of flame angelfish and bluefin trevally larvae were obtained than in any previous studies by us and a small percentage of flame angelfish survived to metamorphosis, a world first. Figure 8 illustrates the increase in mean standard length for both fish species.

While these trials clearly demonstrated the nutritional value of cultured copepods for small tropical marine fish larvae, the onset of chronic mortality during the postlarval phase needs to be addressed, although the present showing provides a sound basis for intensive aquaculture of fish larvae. One explanation for the mortality is that the post-larvae’s digestive capabilities were inadequate for processing rotifers/ *Artemia* and that a longer period of exclusive copepod feeding would be beneficial in this respect. Alternatively, the postlarvae may have encountered adverse microbial conditions following prolonged rearing in the same tank.

The above results demonstrate the feasibility and advantages of rearing fish larvae under controlled conditions where commercial levels of production can be achieved. The processes can use a tank farm where copepods are grown under optimum conditions in a tank and then used to feed fish larvae in a rearing tank, where the process can be substantially continuous and under defined sterile conditions. Using the subject system, the growth of the fish larvae is self-contained and easily controlled. In addition, preferred feeds for the copepods and fish larvae are provided to allow for extended periods of growth, reduced mortality and high efficiencies.

All references referred to in the text are incorporated herein by reference as if fully set forth herein. The relevant portions associated with this document will be evident to those of skill in the art. Any discrepancies between this application and such reference will be resolved in favor of the view set forth in this application.

Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.